

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Himanshu BRAHMBHATT *et al.*

Title: **METHODS FOR TARGETED IN VITRO AND
IN VIVO DRUG DELIVERY TO
MAMMALIAN CELLS VIA BACTERIALLY
DERIVED INTACT MINICELLS**

Appl. No.: 10/588,028

Examiner: Anoop Kumar Singh

Art Unit: 1632

Confirmation
Number: 1320

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

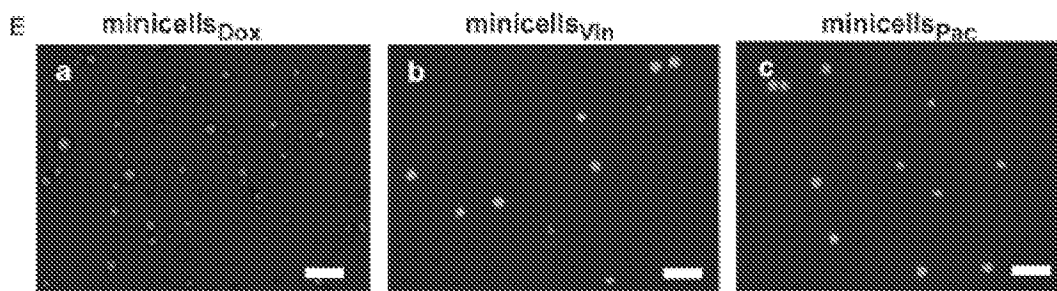
I, Himanshu Brahmbhatt, declare the following:

1. I am a co-founder of EnGeneIC Molecular Delivery Pty Ltd (“EnGeneIC”), and I have served as a Joint-CEO and Director of the company since June, 2004.
2. I received my Ph.D. in 1987 at the University of Adelaide, South Australia. I subsequently carried out my post-doctoral research at the Dept. de Biochemie Medicale, Centre Medicale Universitaire, Geneva, Switzerland (June 1987 - June 1988) and the National Centre for Research in Biotechnology, Braunschweig, Germany (July 1988 - Aug. 1991). I then served as a Research Scientist (Sept. 1991 - June 30th, 1994), a Senior Research Scientist (July 1, 1994 – June 30th, 1999), and a Principal Research Scientist (July 1, 1999 – January 15, 2001) at CSIRO McMaster Laboratory, Division of Animal Health (Sydney, Australia). I have spent much my career studying bacterial vaccines, parasite vaccines, and cancer biology. I have over 12 publications in those and related fields, with most of my research output reflected in various patent applications.

3. I also am a co-inventor named in the captioned application. In that capacity and as a representative of EnGeneIC, I participated in an interview conducted on September 17, 2010, with Examiner Singh, in connection with the application.
4. From that interview I understood that Examiner Singh wished to see evidence concerning the generality, across a range of small molecule drugs, of the invention described and claimed in the application. To that end I provide here data demonstrating that an illustrative number of small molecule drugs can be loaded into minicells and that the resultant, small molecule drug-packaged minicells show significant anti-tumor efficacy. The involved small molecule drugs include:

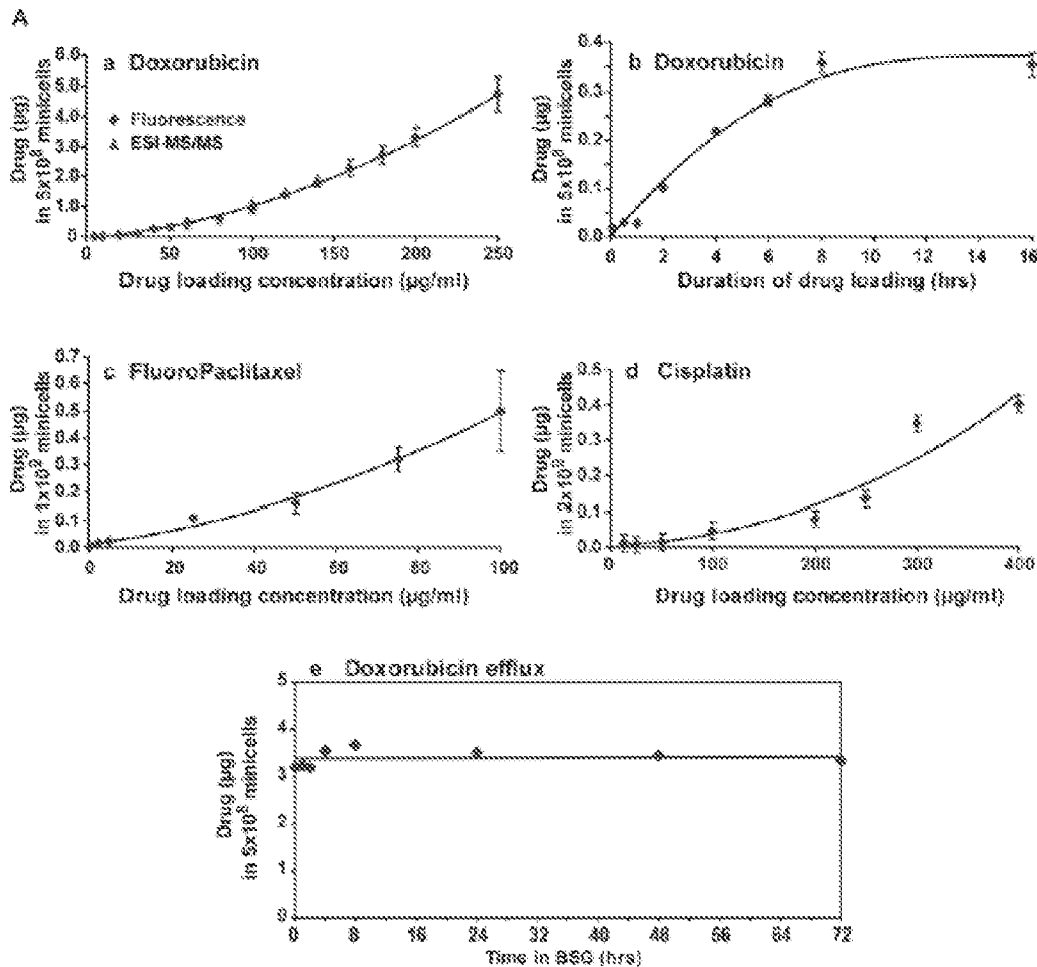
- A. Doxorubicin,
- B. Paclitaxel,
- C. Fluoro-paclitaxel,
- D. Cisplatin,
- E. Vinblastine,
- F. Monsatrol,
- G. Thymidylate synthase (TS) inhibitor OSI-7904
- H. Irinotecan,
- I. 5-Fluorouracil,
- J. Gemcitabine, and
- K. Carboplatin.

A. **Doxorubicin, vinblastine and paclitaxel** – Packaging of doxorubicin, fluorescent vinblastine and fluoro-paclitaxel into intact minicells. These data appear in MacDiarmid *et al.*, *Cancer Cell* 11: 431-45 (2007) (appended). See Figure 1E, reproduced below.



(E) Minicells packaged with Dox, showing red autofluorescence of the drug (Ea), or green fluorescence after loading with either BODIPY FL-conjugated vinblastine (Eb), or Oregon Green 488-conjugated Paclitaxel (Ec). No autofluorescence was observed with empty minicells (data not shown). Scale bar, 5 μ m.

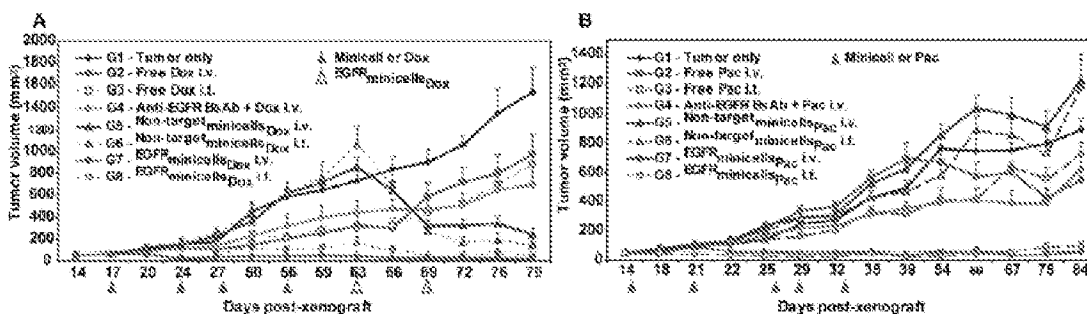
B. Doxorubicin, flouro-paclitaxel and cisplatin – Kinetics of packaging these drugs into minicells demonstrates that the drugs did not leak out of the intact minicells once packaged. See MacDiarmid *et al.*, *Cancer Cell* (2007), at Figure 2A (reproduced below).



This figure quantitates minicell-loading upon incubation in the presence of different drug concentrations (**a, c, d**) and for different times (**b**). In (**a**), (**c**) and (**d**), minicells were incubated for 1 hour with different concentrations of doxorubicin (Dox), flouro-paclitaxel (pac) and cisplatin, respectively. Drug-loading was determined after extraction from minicells, and quantitation by HPLC and fluorescence detection or LC-MS/MS. In (**b**), minicells were incubated with Dox (60 µg/ml) for the times shown ($n = 6$). To evaluate drug efflux after loading, minicells were incubated in an external concentration of Dox (200 µg/ml). The minicells_{Dox} were incubated in BSG for 72 hours at 4°C and Dox was

extracted and quantitated from samples (triplicate) at the time points shown (e). Despite prolonged incubation in BSG, no drug efflux was observed. Error bars; +/- SEM.

C. Doxorubicin and paclitaxel – Human breast cancer xenografts were effectively treated with doxorubicin- or paclitaxel-packaged minicells. See MacDiarmid *et al.*, *Cancer Cell* (2007), at Figures 4A and 4B, *infra*.



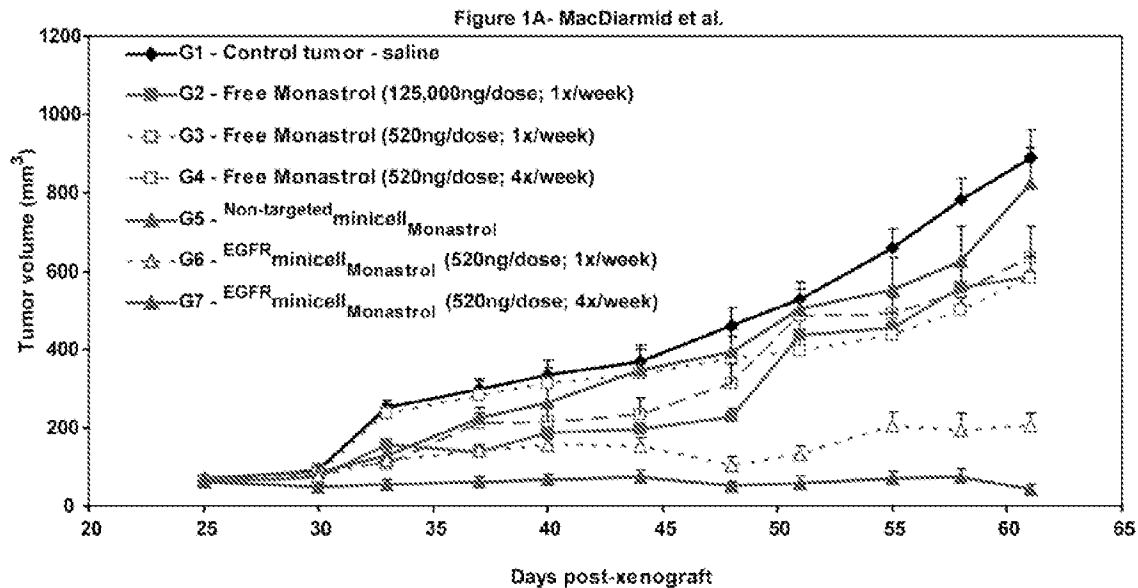
These figures show inhibition/regression of tumor growth in mice with MDA-MB-468 human breast cancer xenografts via EGFR-targeted, paclitaxel- or doxorubicin-packaged minicells. Error bars for all graphs; +/- SEM.

(A) Mice ($n = 11/\text{group}$) bearing MDA xenografts were treated i.v. or i.t. with either free Dox (7.5 $\mu\text{g}/\text{gm}$; $\sim 150 \mu\text{g}/\text{mouse}$), or BsAb (anti-O-antigen/anti-EGFR) plus free Dox (7.5 $\mu\text{g}/\text{gm}$, i.v. only), minicells_{Dox} (10^8), or ^{EGFR}minicells_{Dox} (10^8); the Dox-packaged minicells carrying 0.08 μg Dox per dose. Inhibition of tumor growth is evident by day 56 with ^{EGFR}minicells_{Dox} ($p < 0.0004$ for tumor volume *vs* all other treatments), but not with the other treatments. At day 63, the groups treated with non-targeted minicells_{Dox} were switched to ^{EGFR}minicells_{Dox}-treatment (open blue triangles). This resulted in rapid and highly significant tumor regression ($p < 0.001$ for tumor volume at day 69 *vs* day 63).

(B) The studies were performed as detailed in Figure 4A, except that the animals were given either minicells packaged with Pac (0.05 $\mu\text{g}/\text{dose}$) or free Pac (20 $\mu\text{g}/\text{gm}$; $\sim 400 \mu\text{g}/\text{mouse}$). Inhibition of tumor growth is evident by day 25 in the groups receiving

$EGFR_{minicells_{Pac}}$ ($p < 0.0004$ for tumor volume vs all other treatments), but not in the other groups.

D. Monastrol – Human breast cancer xenograft effectively treated with monastrol-packaged minicells. These data are published in MacDiarmid *et al.*, *Cell Cycle* 17: 1-7 (2007) (appended). See Figure 1A below.



This figure depicts inhibition/regression of tumor growth in mice treated with receptor-targeted minicells packaged with molecularly targeted anti-cancer drugs. Human breast cancer (MDA-MB-468) xenografts in Balb/c *nu/nu* mice ($n = 8$ per group) treated with free monastrol (G2 to G4), $non-targeted_{minicell_{Monastrol}}$ (G5) or $EGFR_{minicell_{Monastrol}}$ (G6 and G7). All doses were administered via a tail vein injection. All minicell treatments received 10^8 minicells per dose. The result shows mean tumor volume (y-axis) in various groups of mice vs. days post-establishment of tumor xenografts (x-axis).

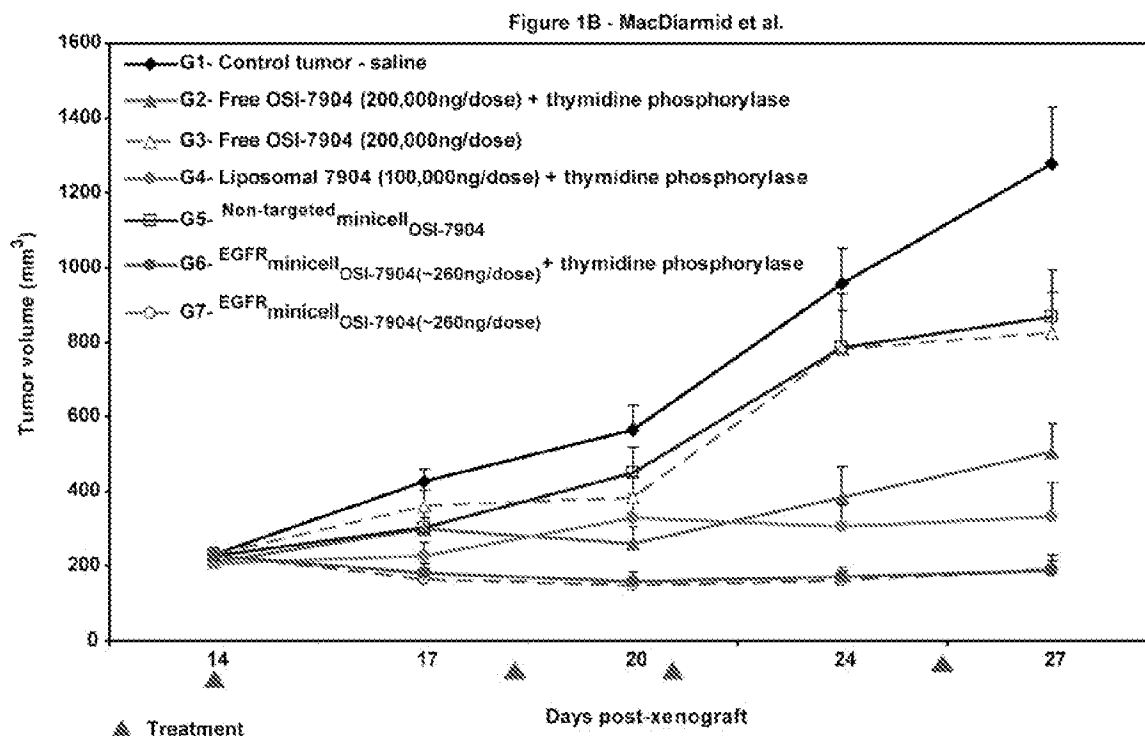
The kinesin spindle protein (KSP), also termed kinesin-5 or Eg5, is a microtubule motor protein that is essential for the formation of bipolar spindles and the proper segregation of sister chromatids during mitosis. Inhibitors of KSP, like Monastrol, cause the formation of monopolar mitotic spindles, activates the spindle assembly checkpoint, and arrests cells at mitosis, which leads to subsequent cell death.

In order to determine whether a targeted minicell vector could package and enhance the therapeutic index of a weak molecularly targeted drug like Monastrol, we carried out an experiment where a human breast cancer xenograft was established in nude mice and treated with EGFR-targeted, monastrol-packaged minicells (EGFR minicells_{Monastrol}) and compared the anti-tumor effects with the administration of free monastrol. LC-MS/MS results showed that 10^8 EGFR minicells_{Monastrol} carried ~520 ng of the drug. Balb/c *nu/nu* mice were purchased from Animal Resources Centre (Perth, WA, Australia), and all animal experiments were performed in compliance with the guide of care and use of laboratory animals and with Animal Ethics Committee approval. Human breast adenocarcinoma cells (MDA-MB-468, ATCC; human mammary epithelial cells) were grown and established as a xenograft between the shoulder blades of each mouse and tumor volume was measured twice a week. 18 days post-implantation, the tumors reached ~80 mm³, and mice were randomized to seven different groups ($n = 8$ per group).

EGFR minicells_{Monastrol} treatment of the mice was compared with non-targeted minicells_{Monastrol} and free monastrol treatments as shown in the figure. The results showed a highly significant anti-tumor effect with EGFR minicells_{Monastrol} treatment (G7 vs G1 to G5; $p < 0.0004$) while free monastrol and non-targeted minicells_{Monastrol} showed no anti-tumor effects.

The highly significant anti-tumor effects with EGFR minicells_{Monastrol} was observed notwithstanding a 240-fold lower dose of Monastrol relative to free drug treatment (compare groups G6 or G7 vs G2).

E. Thymidilate synthase inhibitor OSI-7904 – Human colon cancer xenografts were effectively treated with thymidilate synthase inhibitor OSI-7904-packaged minicells. See MacDiarmid *et al.*, *Cell Cycle* (2007), at Figure 1B (reproduced below).



This figure illustrates that human **colon cancer** (HT29) xenografts in Balb/c *nu/nu* mice ($n = 8$ per group) were administered i.v. with the various treatments shown in the figure. All minicell treatments received 10^8 minicells per dose. Treatment days are shown below the x-axis (red triangles). Error bars for both graphs; \pm SEM.

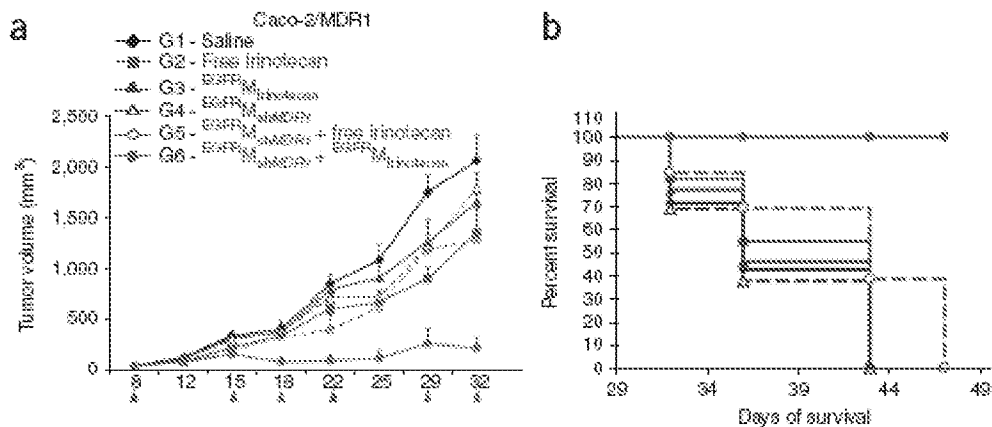
Thymidylate synthase (TS) inhibitors form another class of new anti-cancer targeted drugs in development. OSI-7904 [(S)-2-[5-[1,2-dihydro-3-methyl-1-oxobenzo[f]quinazolin-9-yl)methyl]amino-1-oxo-2 isoindolynl]-glutaric acid] is a potent selective non-competitive TS inhibitor. We carried out a xenograft study in nude mice ($n = 8$ mice per group) to compare the anti-tumor effects of free OSI-7904, liposomally encapsulated OSI-7904 (OSI7904L; both a kind gift from Niel Gibson, OSI Pharmaceuticals Inc., Melville, NY, USA) and either EGFR-targeted or non-targeted minicells packaged with OSI-7904 (designated ^{EGFR}minicells_{OSI-7904} and minicells_{OSI-7904}, respectively). Since circulating levels of thymidine in rodents is relatively high, it can ameliorate the cytotoxicity of TS inhibitors. To bypass the thymidine salvage pathway, efficacy studies in rodents are often performed by intraperitoneal (i.p.) administration of thymidine phosphorylase, which lowers circulating thymidine levels by metabolizing thymidine to thymine and deoxyribose-5-

phosphate. Therefore, in this study we also included additional treatment groups of free OSI-7904, OSI-7904L and ^{EGFR}minicells_{OSI-7904} where thymidine phosphorylase was administered i.p. (Groups 2, 4 and 6, respectively).

The study was carried out in HT29 human colon cancer xenografts and tumors were allowed to grow to 200 mm³ to 250 mm³ before the various treatments were administered i.v. via the tail vein.

The results showed a highly significant anti-tumor effect following ^{EGFR}minicells_{OSI-7904} treatment (G7 mice). Additionally, the anti-tumor effects were identical in mice treated with ^{EGFR}minicells_{OSI-7904} or ^{EGFR}minicells_{OSI-7904} with thymidine phosphorylase (G7 and G6 mice respectively). This is in contrast to the groups treated with free OSI-7904 where some reduction in tumor growth rate was only seen in the thymidine phosphorylase pre-treated group (G2 vs G3 mice). Presumably, since the drug was encapsulated in the minicells and only released intracellularly, the ^{EGFR}minicells_{OSI-7904} treatment may not be subject to circulating levels of thymidine and hence the thymidine salvage. OSI-7904L formulation was effective in stabilizing tumour growth but not as effective as ^{EGFR}minicells_{OSI-7904}. More importantly, ^{EGFR}minicells_{OSI-7904} was more effective (G7 mice, 260 ng drug/dose) at a dose that was ~385-fold less than the liposomal formulation OSI-7904L (G4 mice, 100,000 ng drug/dose). The minicell delivery vector thus dramatically increased the therapeutic index.

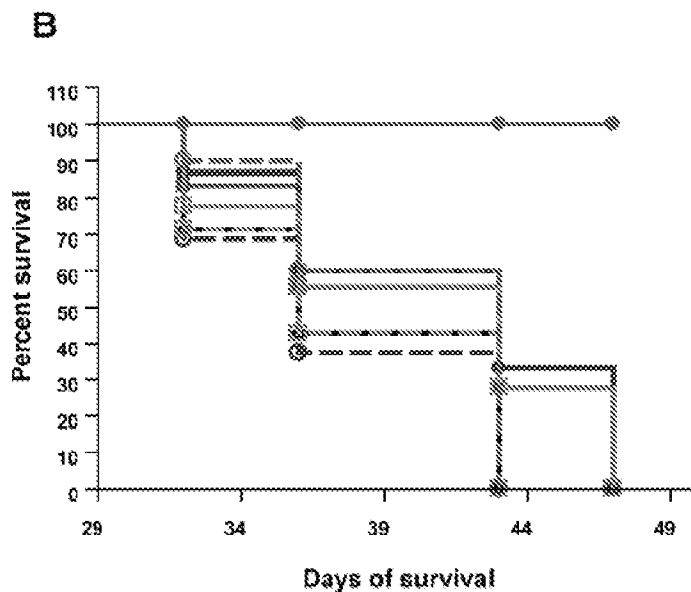
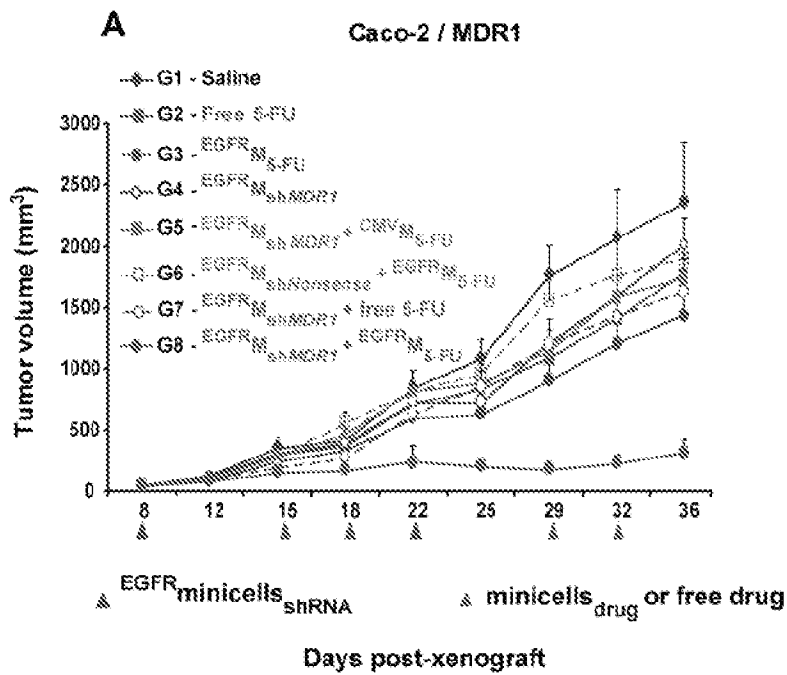
F. Irinotecan – Effective treatment of irinotecan-resistant human colon cancer xenografts following dual sequential treatment with shRNA-MDR1-packaged minicells followed by irinotecan-targeted minicells. These data have been published in MacDiarmid *et al.*, *Nature Biotechnology* 27: 643-51 (2009) (appended). See Figures 5a and 5b, below.



To investigate whether dual minicell therapy, both to reverse drug resistance and to inhibit tumor growth, is effective as an *in vivo* therapeutic approach, Caco-2/MDR1 colon cancer xenografts were treated with ^{EGFR}minicells_{shMDR1} followed by either free irinotecan or ^{EGFR}minicells_{Irinotecan}. In initial studies, we determined that the optimal time between treatment with siRNA or shRNA-packaged minicells to effect knockdown of the drug resistance protein, MDR1, and then treatment with cytotoxic drug-packaged minicells was ~48 hours and ~144 hours, respectively.

As shown in the figure, compared to all control groups, dual sequential treatment with ^{EGFR}minicells_{shMDR1} followed by ^{EGFR}minicells_{Irinotecan} given on the days indicated, produced marked inhibition of tumor growth (G6 vs all controls, $p < 0.0001$), despite the xenografts being highly resistant to free irinotecan (G2). A Kaplan-Meier survival analysis showed 100% survival of the mice treated with the dual treatment procedure while those in all other groups died early. This marked survival difference occurred despite irinotecan being given at 12 mg/kg per dose (~240 µg per mouse dose), whereas the equivalent minicell mouse dose was ~80 ng, equating to ~3,000-fold less irinotecan being administered via minicell-delivery.

G. 5-Fluorouracil – Effective treatment of 5-Fluorouracil-resistant human colon cancer xenografts following dual sequential treatment with shRNA-MDR1-packaged minicells followed by 5-Fluorouracil-targeted minicells. See MacDiarmid *et al.*, *Nature Biotechnology* (2009), Supplementary Figures 4A and 4B, *infra*.

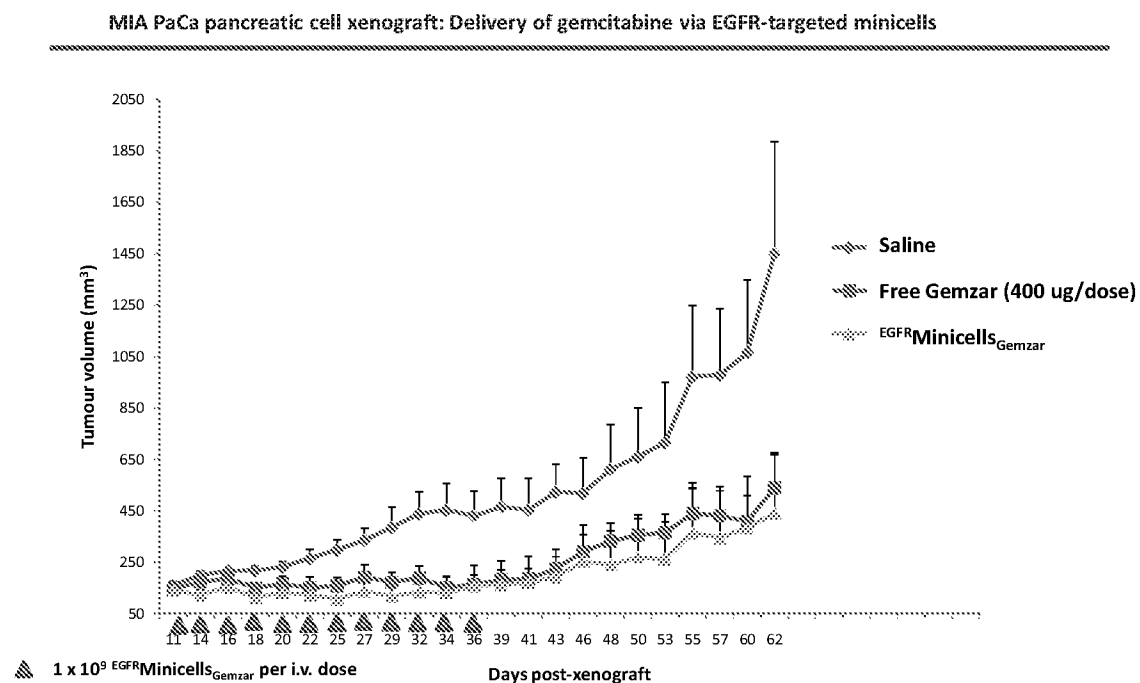


Minicells (10^9 per dose) were administered i.v. to mouse xenografts. The concentrations of drugs or shRNAs administered per minicell dose were: **(a)** ~100 copies of shRNA, and **(b)** ~40 ng 5-FU (5-Fluorouracil). Free 5-FU was administered at a dose of 1,000 μ g. The days of treatment administration of the various minicells are shown below the x-axis. Error bars indicate \pm S.E.M.

(A) Drug resistant Caco-2/MDR1 xenografts ($n = 6/\text{group}$) were treated with $\text{EGFR}^{\text{minicells}}_{\text{shMDR1}}$ followed by $\text{EGFR}^{\text{minicells}}_{5\text{-FU}}$, which produced highly significant ($p < 0.0001$ vs all controls at day 36) anti-tumor effects. The various control treatments are shown in the graph. $\text{CMV}^{\text{minicells}}_{5\text{-FU}}$ (control) carries a BsAb that is directed to an irrelevant antigen, CMV, cytomegalovirus capsid protein.

(B) Kaplan-Meier survival analysis over 47 days of the animals treated in (A) showing 100% survival only in the mice receiving the dual treatment of $\text{EGFR}^{\text{minicells}}_{\text{shMDR1}}$ followed by $\text{EGFR}^{\text{minicells}}_{5\text{-FU}}$ (G8 mice).

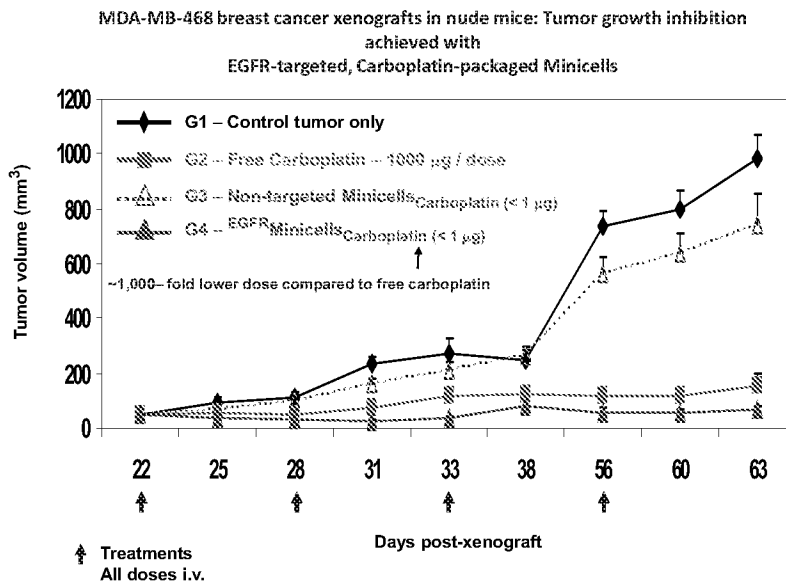
H. **Gemcitabine** – Human pancreatic cancer xenografts were effectively treated with Gemcitabine (Gemzar®)-packaged minicells (unpublished data).



Human pancreatic cancer (MIA PaCa) xenografts in Balb/c *nu/nu* mice ($n = 8$ per group) were administered i.v. with either free Gemzar or EGFR-targeted, Gemzar-packaged minicells ($\text{EGFR}^{\text{minicells}}_{\text{Gemzar}}$). All minicell treatments received 10^9 minicells per dose. Treatment days are shown below the x-axis (blue triangles). Error bars for both graphs; +/- SEM. The results show that although the minicell doses carried ~ 50 ng of Gemzar, the anti-

tumor efficacy of EGFR Minicells_{Gemzar} treatments were just as effective in terms of anti-tumor efficacy as free Gemzar that was given at a dose of 400,000 ng per dose.

I. Carboplatin – Human breast cancer xenografts were effectively treated with carboplatin-packaged minicells (unpublished data).



Human breast cancer (MDA-MB-468) xenografts in Balb/c *nu/nu* mice ($n = 8$ per group) were administered i.v. with either free carboplatin or non-targeted minicells packaged with carboplatin or EGFR-targeted, carboplatin-packaged minicells (EGFR Minicells_{Carboplatin}). All minicell treatments received 10^9 minicells per dose. Treatment days are shown below the x-axis (blue arrows). Error bars for both graphs; \pm SEM. The results show that EGFR Minicells_{Carboplatin} treatments were highly effective in achieving tumor stabilization, even though the dose of carboplatin was $\sim 1,000$ -fold lower than the free carboplatin dose.

5. I declare that the statements made herein of my knowledge are true and all statements on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code,

and that such willful false statements may jeopardize the validity of the application or any patent issuing therein.

6th December 2010
Date

Himanshu Brahmabhatt
Himanshu Brahmabhatt